Low molecular weight heparin prevents CLP-Induced acute lung injury in rats by anti-inflammatory coagulation

Xiao Lu[1*], Liang Zhao[2], Yong-Hua Xu[2]

[1] ER Department the 2nd Affiliated Hospital of Zhejiang University School of Medicine,No.88 Jiefang Road Hangzhou 310011, China.
[2] ICU Department Shanghai ChangZheng Hospital, No 440 Chendu Road Shanghai 200003, China

Abstract

The aim of our study was to observe the influence of low molecular Weight heparin (LMWH) on systemic inflammation, including high mobility group box 1 protern (HMGB1) and protective effect on acute lung injury induced by cecal ligation and puncture(CLP). Discuss the mechanism of this effect. 144 male SD rats were randomly divided into sham operation group (A), normal treatment group (B), the LMWH treatment group (C), n=48.Group A received a sham operation and the other groups were underwent CLP operation. Groups A and B accepted intraperitoneal injection (i.p.) of normal saline (NS) at a dose of 2.0ml/kg and ceftriaxone (30 mg/kg), Group C were intraperitoneal injection additional LMWH (150U/kg) except saline and ceftriaxone. Observe points were made at 3, 6, 12, 18, 24, 48 h, the rats were anesthetized and killed, mortality, lungs wet/dry ratio and Pathology change were determined. HMGB-1 mRNA, protein of lung tissues was calculated by RT-PCR and Western blot. TNF-α and IL-6 of blood plasma calculated by ELSIA. There was significantly different in each index between A and B group (p<0.05).Compared with CLP group, there was a significant decrease in the lung injury, the mortality, HMGB1 mRNA and protein expression on lung tissues (p<0.05). LMWH can decreases cytokine, HMGB1 levels of lung tissue during CLP-induced inflammation. As a result, LMWH ameliorated lung pathology and reduces mortality in CLP-induced systemic inflammation in a rat model. This effect may be mediated through the inhibition of axis of inflammation and coagulation.

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KEY WORDS: HMGB-1, low molecular weight heparin, sepsis, lung injury

INTRODUCTION

Since the concept of the sepsis has been put forward in 1991, although there are less progress in the research of sepsis outbreak mechanism. The anti-inflammatory treatment of sepsis in clinal can not reduce the mortity expect active protein C, this result make us to think highly of the important of the anticoagulation in the sepsis treatment. Recently, it has been demonstrated that the high mobility group box 1 (HMGB1) protein plays a key role as a late-phase mediator of lipopolysaccharide (LPS) lethality and systemic inflammation [1]. HMGB1 is constitutively expressed in many cell types and is stored in the nucleus due to the presence of two lysinerich nuclear localization sequences [2]. Extracellular HMGB1 plays a pathogenic role in infection or injury elicited inflammatory diseases. HMGB1 is first detectable in the circulation 8h after onset of lethal endotoxemia and sepsis, and subsequently increases to plateau levels from 18 to 24h [3]. HMGB1 acts as a pro-coagulant [4], thereby enhancing the inflammatory response in septic shock [5, 6]. Inhibitors of HMGB1 might therefore be beneficial in the treatment of various inflammatory diseases. This study is to observe the influence of low molecular Weight heparin on systemic inflammation, including the high mobility group box 1 protern(HMGB1) and protective effect on acute lung injury induced by cecal ligation and puncture(CLP).

MATERIAL AND METHODS

Animals

The study was approved by the second mililary medicial universily, shang hai, china. Male Wistar rat weighting 200 to 300 g(Experiment Animal Center of second mililary medicial universily,Shang Hai.China) were used.Anaesthesia was induced by 4% sevoflurane.The animal were randomly assigned to one of three group.(1) Negative control group: rats treated with 0.9 % NaCl solution (1.0ml/kg) and ceftriaxone (30 mg/kg) after surgery (open the abdomen, find the cecum, then turn back), animal had unlimited access to food and water. (2) normal treated CLP group: rats treated with 0.9 % NaCl

* Corresponding author: Xiao Lu,
ER Department the 2nd Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 300011, China
Tel:+86-571-88354296
e-mail: drluxiao@163.com

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solution (1.0 ml/kg) and ceftriaxone (30 mg/kg) after CLP; (3) LMWH-treated CLP group rats: rats treated with 0.9%NaCl solution (1.0 ml/kg), LMWH 150U/kg and ceftriaxone (30 mg/kg) after CLP.

Materials

Danaparoid sodium was purchased from Pfizer Co.Ltd (Shanghai, China), ceftriaxone was purchased from Roche Co.Ltd(Shanghai,China) ,Antibodies to rabbit polyclonal Ig E anti-HMGB1 were purchased from Abcam (USA).

Histological analysis

A pathologist blind to group assignment analysed the samples and determined levels of lung injury according to Murakami's technique. Briefly, 24 areas in the lung parenchyma were graded on a scale of 0 to 4 (0, absent and appears normal; 1, light; 2, moderate; 3, strong; 4, intense) for congestion, oedema, infiltration of inflammatory cells, and hemorrhaging.

Measurements of cytokine secretion

IL-6 and TNFα levels were determined using a commercial enzyme-linked immunosorbent assay kit. IL-6 and TNFα were from R&D Systems Inc, Minneapolis, MN, USA.

Western blotting

Proteins were subjected to SDS-PAGE, and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibody (1:1,000 dilution). After incubation with secondary antibody, blots were developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm ECL (Amersham). Weused the NIH Image J software (National Institutes of Health, Bethesda, MD, USA) to quantitate protein band concentrations.

Detection of lung HMGB1 mRNA with RT-PCR

Lung tissue weighing approximately 50 mg was collected with aseptic technique. Single step method was applied for extracting total RNA from pneumonocytes with acid guanidinium thiocyanate-phenol-chloroform extraction. The specimens of rat lung tissue were subjected to semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) amplification with the primers of HMGB-1 (forward primer: 5'-ATG GGC AAA GGA GAT CCT A-3'; reverse primer: 5'-ATT CAT CAT CAT CAT CTT CT-3',expected to amplify rat HMGB-1 cDNA of about 680 bp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes (forward primer: 5'-TCC CTC AAG ATT GTC AGC AA-3'; reverse primer: 5'-AGA TCC ACA ACG GATACATT-3', with amplified fragment length of 309 bp, served as inter-reference). The amplified product was preformed 20 g/l agarose (20 g/l) gel electrophoresis. After electrophoresis, the gel is placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern is taken with a Polaroid camera, and then integral absorbance (Ai) was calculated. Ratio of Ai (target gene) to Ai (inter-reference)was regarded as relative amount of HMGB1 gene expression.

Determination of the wet/dry ratio.

The lung tissue was cleaned of blood stains with absorbent paper, weighed wet, torrefied in a 75 °C thermostatic baking oven for 72 hours, and weighted again dry to calculate the lung D/W weight ratio.

Statistical analysis

For descriptive purposes, all continuous data were presented as mean ± SD. The data were analysed by Mann-Whitney U test for comparison between two independent groups. A p value of less than 0.05 was considered to be statistically significant. Survival data were analyzed with the Kaplan-Meier program included in the Prism 4.0 software package (San Diego, CA, USA). p Values less than 0.05 were considered statistically significant.

RESULTS

Mortality

A total of 60% of the rats died within 12h in CLP group, and an additional 15% died within 24h, all rats died in 5 days, while only 10% rats in the LMWH-treated CLP

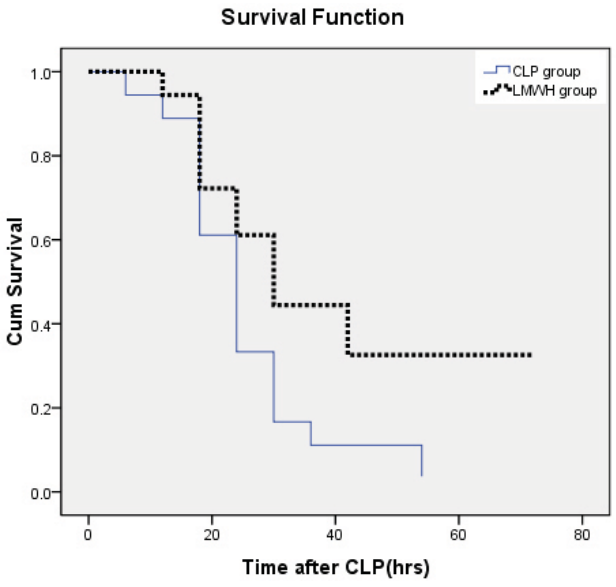


FIGURE 1. Kaplan-Meier analysis revealed a significantly shorter time-to-death among the CLP group compared to the LMWH-treated CLP group (p<0.05).

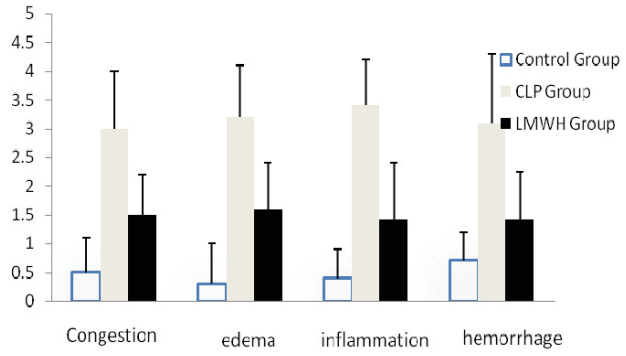


FIGURE 2. Effects of LMWH on lung histopathology score in CLP-administered rats. The histological changes identified included congestion, oedema, inflammation, and haemorrhaging 12 h after the CLP. The data are expressed as the mean ± SD. *Denotes a significant difference compared with the LPS group ($p < 0.05$).

group (150U/L) died. All of control group animal survival for 5 days. Kaplan-Meier analysis revealed a significantly shorter time-to-death among the CLP group compared to the LMWH-treated CLP group ($p<0.05$).

Effects of LMWH on Lung Tissue

Microscopic observation of lung tissue samples taken 6 h after CLP showed interstitial edema and infiltration by neutrophils in both the CLP and LMWH +CLP groups. No histological alterations were seen in the control group. The LMWH +CLP group showed markedly reduced interstitial edema and inflammatory cell infiltration in comparison to the CLP group. The histology scores, based on the number of areas with congestion, edema, Inflammation,

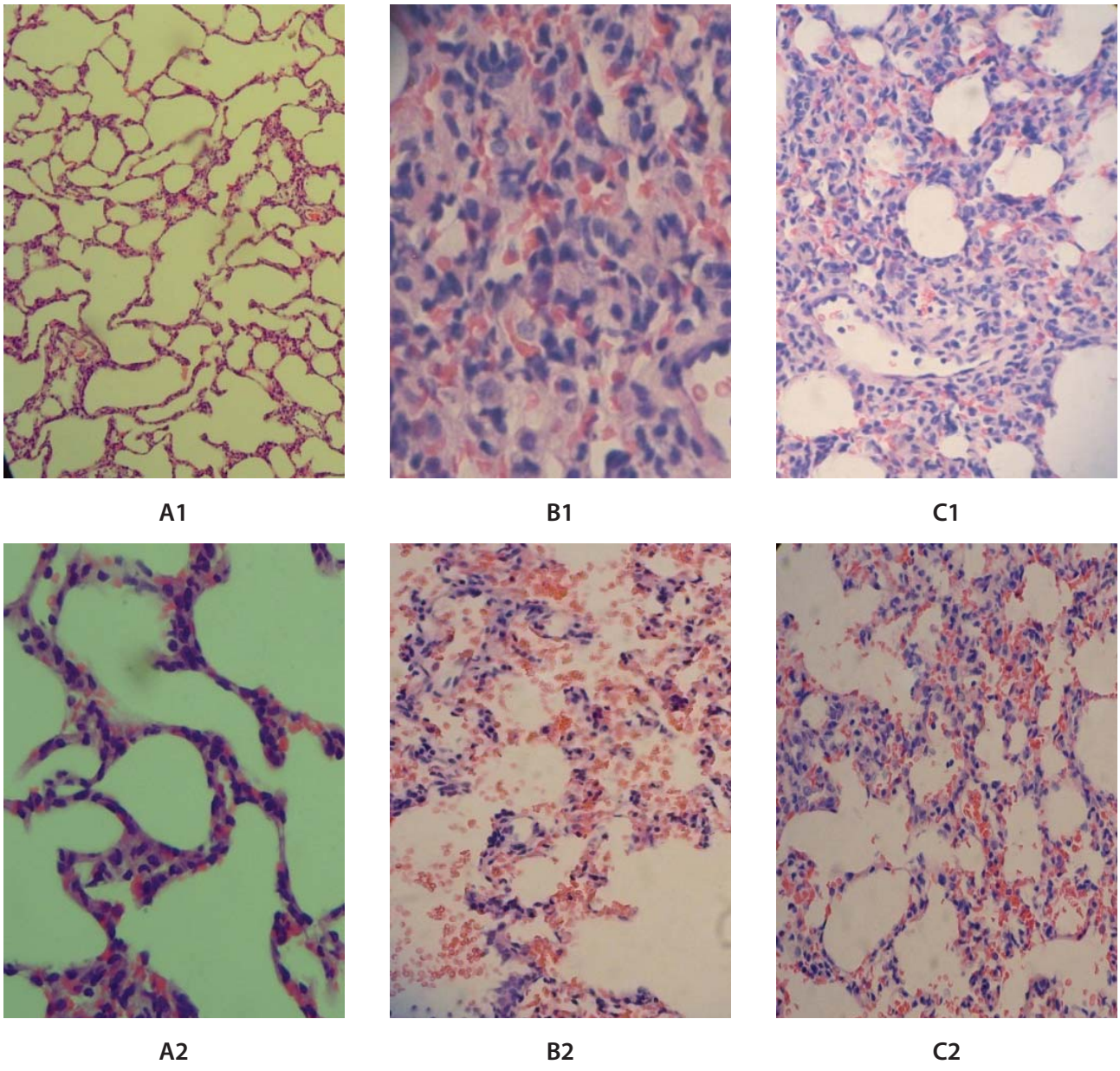


FIGURE 2. Effect of LMWH on lung histopathology in septic rats induced by CLP.Lung tissue specimens were obtained from negative control(A1)magnificationx40,(A2) magnificationx100;CLP group (B1) magnification x40,(B2) magnification x100; LMWH group (C1) magnification x40, (C2) magnification x100. Haematoxylin and eosin staining was used.

and hemorrhaging, were all significantly higher after the administration of CLP than in the control group. All of the scores were lower in the group pretreated with LMWH.

Comparison of Lung D/W Ratio

The lung D/W ratios in three Groups were 4.074±(0.524), 6.12±(0.991) and 4.42±(0.162), respectively (p<0.01). The LSD test indicated that the lung D/W ratio in CLP group and LMWH group was higher than that in control Group (p<0.05). LMWH group was significantly lower than that in CLP group (p<0.05).

Effects of LMWH on the Serum Levels of IL-6, TNF-α

Before CLP, IL-6 and TNF-α were not detected in serum samples from any of the groups. Serum levels of IL-6 peaked 3h following CLP in both CLP and LMWH + CLP groups. The IL-6 levels in the serum were significantly lower at 6, 9, and 12h following CLP in the LMWH + CLP group than in the CLP group Serum levels of TNF-α also peaked 3 h after the CLP. The TNF-α levels were significantly lower3 h after CLP in the LMWH + CLP group compared with the CLP group, but were not significantly different at later time points.

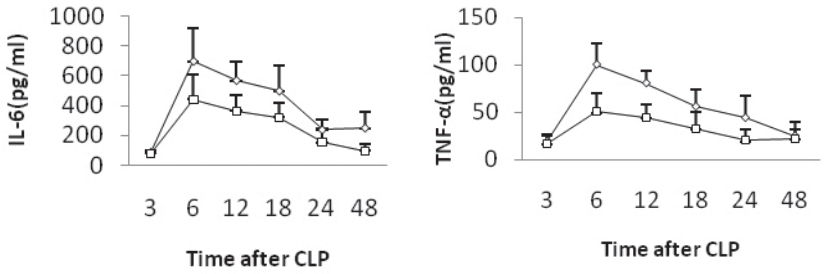


FIGURE 4. Effects of LMWH on the Serum Levels of IL-6, TNF-α.Serum levels of IL-6 peaked 3h following CLP in both CLP and LMWH + CLP groups. The IL-6 levels in the serum were significantly lower at 6, 9, and 12h following CLP in the LMWH + CLP group than in the CLP group Serum levels of TNF-α also peaked 3 h after the CLP. The TNF-α levels were significantly lower 3h after CLP in the LMWH + CLP group compared with the CLP group, but were not significantly different at later time points.

Effects of LMWH on the HMGB1 Levels in Lung Tissue Specimens after the CLP

HMGB1 was slightly expressed in lung tissue of the control group 12 h after saline administration. In contrast, lung tissue from the CLP group showed increased HMGB1 expression 12 h after CLP. The pretreatment of rats with LMWH prior to CLP led to a decrease in the expression of HMGB1 in lung tissue compared to the CLP group. HMGB1 levels did not significantly change in the control group during the experiment.

Pulmonary tissue HMGB1 mRNA and protein expression

HMGB1 mRNA expression was rare in pulmonary tissue of normal rats. Compared with that of control group, the septic rat lung tissue HMGB1 mRNA expression was upregulated significantly 6–24 h after CLP exposure (p <0.05); LMWH intervention remarkably ame-

TABLE 1. The expression of HMGB1 mRNA in lung tissue of septic rats (n=5, $\bar{X}$ ±s)

group	3h	6h	12h	18h	24h	48h
A组	0.038 ± 0.029	0.034 ± 0.031	0.038 ± 0.032	0.041 ± 0.054	0.044 ± 0.038	0.042 ± 0.012
B组	0.049±0.025	0.061±0.024	0.142± 0.084##△△++	0.351± 0.062##△△++▲	0.472± 0.012##△△++▲○○	0.461± 0.078##△△++▲▲○○◆◆
C组	0.040±0.064	0.033±0.098	0.092± 0.072##★△△++	0.155± 0.034##★★++▲▲	0.258± 0.033##★★++▲▲○○	0.236± 0.067##★★++▲▲○○◆◆

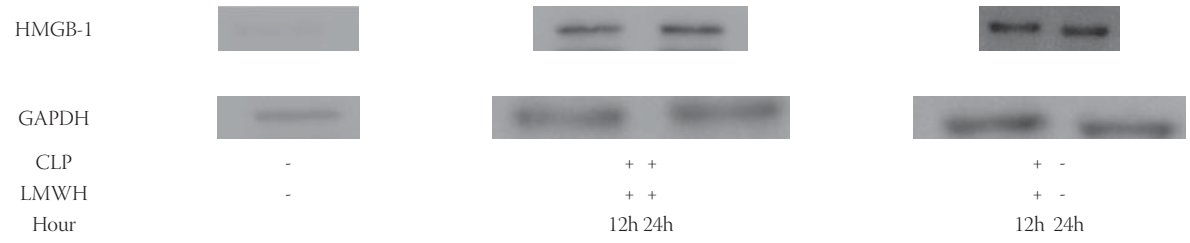
Compare to A group at the same time point: ## p<0.01；Compare to B group at the same time point：★ p<0.05，★★ p<0.01；Compare to same group at 3h：△△ p<0.01；Compare to same group at 6h：++ p<0.01；Compare to same group at 12h：▲p<0.05，▲▲ p<0.01；Compare to same group at 18h：○○ p<0.01；Compare to same group at 24h：△△ p<0.01

TABLE 2. The expression of HMGB1 Protein in lung tissue of septic rats (n=5, $\bar{X}$ ±s)

Group	3h	6h	12h	18h	24h	48h
A	0.561± 0.119	0.505 ± 0.031	0.702± 0.132	0.803± 0.341	0.881± 0.784	0.902± 0.521
B	1.103± 0.837	1.003 ± 0.663	1.562± 0.695##△△++	1.654± 1.547##△△+▲	2.883± 0.866##△△++▲▲○○	2.782± 1.047##△△+▲▲○○◆
C	0.532± 0.287	0.605± 0.511	1.246± 0.439##★△△++	1.345± 1.102##★★+▲	1.572± 1.033##★★++▲○○	1.779 ±1.132##★★++▲▲○○◆

Compare to A group at the same time point: ##p<0.01；Compare to B group at the same time point：★p<0.05，★★ p<0.01；Compare to same group at 3h：△△p<0.01；Compare to same group at 6h：++ p<0.01；Compare to same group at 12h：▲p<0. 05，▲▲p<0. 01；Compare to same group at 18h：○○ p<0.01；Compare to same group at 24h：△△p<0.01

TABLE 3. Effect of LMWH on the HMGB-1 protein expression level in Lung Tissue Specimens of septic rats



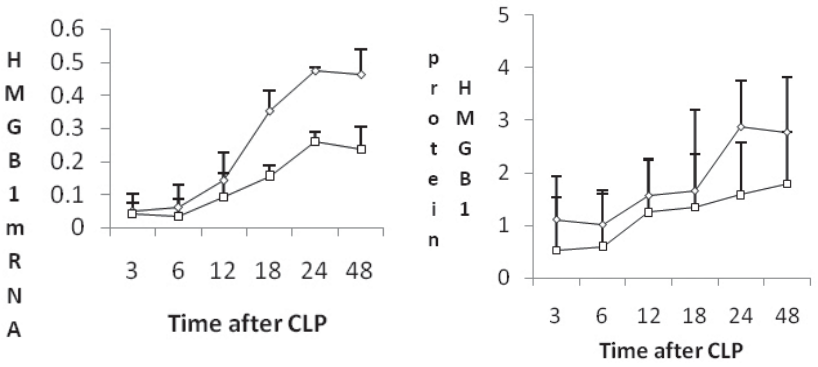


FIGURE 5. Pulmonary tissue HMGB1 mRNA and protein expression. HMGB1 mRNA expression was rare in pulmonary tissue of normal rats. Compared with that of control group, the septic rat lung tissue HMGB1 mRNA expression was upregulated significantly 6–24 h after CLP exposure (P <0.05); LMWH intervention remarkably ameliorated the upregulation. The difference of HMGB1 mRNA expression was significant between CLP group and LMWH group (P<0.05) (Fig. 1; Table 1).CLP results in increased high mobility group box 1 (HMGB1) expression in the lung.

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DISCUSSION

The model of cecal ligation and puncture (CLP) in rodents has been used extensively to investigate the clinical settings of sepsis and septic shock. This model produces a hyperdynamic, hypermetabolic state that can lead to a hypodynamic, hypometabolic stage, and eventual death. Blood cultures are positive for enteric organisms very early after CLP [7]. Compared to lipopolysaccharide inject model, CLP model can simulate the progress of polymicrobial sepsis which is more similar to the progress of sepsis in clinal [8]. As such, we selected a CLP-induced ALI animal model to evaluate inflammatoty and coagulation pathways following the administration of LMWH. Severe sepsis is a common disease and often complicated with ALI. This study is to demonstrate the anti-inflammatory actions of LMWH in a rat model of CLP-induced lung injury. Acute inflammatory events, such as those that occur in ALI, lead to dysregulation of the coagulation cascade. Indeed, ALI is characterised by profound alterations in both systemic and intra-alveolar coagulation and fibrinolysis [9]. Activation of coagulation with resultant fibrin deposition also has proinflammatory consequences, serving to further amplify the inflammatory cascade [10]. So it suggest there will be effective using the anticoagulant in therapying the ALI induced by sepsis. Previous studies have reported that HMGB1 is critical for inflammation and development of septic lung injury [11-13]. HMGB1 has played an important role in the process of sepsis as a later mediator of inflammation, its expression level is highly relevant to severity degree and prognosis of septic rats [14, 15]. The study has also proved there is some effect in the coagulation and microthrombus during the sepsis [16], which is equal to the cross talk of inflammation and coagulation in the process of sepsis. We believed that the anti-inflammatory and anticoagulation effect through the inhibition of HMGB1 could improve the treatment effectiveness of sepsis. It has been proved that HMGB1 is a high affinity binding protein of heparin, which inhibits the HMGB1 by change its conformation. Our study has showed also that the high level of the HMGB1 in the lung issue at the time after the CLP 12h-24h. Compared to those early media of inflammation such as IL-1β, IL-6 and TNF-α, HMGB1 could give us a wide treatment window of sepsis which has more significance [17]. Extensive in vitro studies have revealed that activation of PAR1 on numerous cell types, including among others fibroblasts, epithelial cells, monocytes/macrophages and vascular endothelial cells, leads to the induction and release of potent pro-inflammatory mediators and chemokines [18]. PARs may play a central role in perpetuating the interplay between coagulation and inflammation. Moreover, thrombin has also been reported to induce the expression of endothelial cell adhesion molecules, including P-selectin and intercellular adhesion molecule-1 (ICAM-1) in vitro and may therefore facilitate the recruitment of inflammatory cells via the production of chemokine networks and upregulation of adhe-

sion molecule expression. PAR1 is also the major thrombin receptor expressed on microvascular endothelial cells, and activation of PAR1 by thrombin promotes endothelial cell permeability and contraction in vitro. Wide spread microvascular injury and leak are common features of ALI/ARDS [19]. In this study, we demonstrated that CLP-induction of PAR1 was dramatically reduced with treatment of LMWH. Our findings suggest that the decrease in PAR1 expression might be directly or indirectly due to decreased inflammation. Impairment of the protein C pathway plays a central role in the pathogenesis of sepsis. Administration of recombinant human activated protein C (rhAPC) may correct the dysregulated anticoagulant mechanism and prevent propagation of thrombin generation and formation of microvascular thrombosis. Furthermore, it may simultaneously modulate the inflammatory response. It is likely that the beneficial effect of rhAPC observed in experimental and clinical studies of severe sepsis results from a combination of mechanisms that modulate the entangled processes of coagulation and inflammation. Heparin also has the combined mechanisms of anticoagulation and anti-inflammation for the treatment of ALI. LMWH is relatively inexpensive, but rhAPC is costly. In the developing countries like China, LMWH may be an attractive alternative to rhAPC, but its therapeutic effect needs to be confirmed by cell-based studies and larger clinical studies. Our findings indicate that LMWH administration could thus inhibit not only early cytokines in the serum but also HMGB1 in lung tissue. Through the inhibition of HMGB1 levels in rats, LMWH may therefore inhibit the inflammatory response and alleviate lung injury symptoms by inhibiting the cross talk between the inflammation and coagulation. Lung damage may result not only from the release of inflammatory mediators, but also from coagulation. These results suggest that coagulation and inflammation are related and therefore, anticoagulant therapy, such as treatment with LMWH, will benefit patients with ALI. In this study, we demonstrated that treatment with the anticoagulant LMWH significantly improved acute lung injury and mortality in a rat model of CLP.

CONCLUSION

In the present study, we demonstrated that LMWH reduced CLP-induced lung injury and reduced PAR-1, HMGB1 levels in lung tissue, reduce pulmonary histopathology, decrease mortality, and diminish systemic inflammatory mediators. These data suggest that LMWH suppresses the activation of the cytokine network, thereby inhibiting the production of HMGB1 and reducing CLP-induced lung injury in rats. In conclusion, our results suggest that LMWH has a therapeutic effect on septic rats not only because of its anticoagulant activity but also because of its ability to inhibit HMGB1 production. Such properties of LMWH might therefore be useful for treating patients with sepsis by reducing pulmonary pathologyas well as coagulation abnormalities. Further research is needed to better explore this possibility.

DECLARATION OF INTEREST

The author declared that he had no conflicts of interest with respect to his authorship or the publication of this article.

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